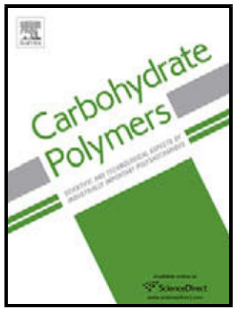


Title: High Concentration Honey Chitosan Electrospun Nanofibers: Biocompatibility and Antibacterial Effects

Author: Wessam A. Sarhan Hassan M.E. Azzazy

PII: S0144-8617(14)01247-8
DOI: http://dx.doi.org/doi:10.1016/j.carbpol.2014.12.051
Reference: CARP 9554

To appear in:

Received date: 2-10-2014
Revised date: 12-12-2014
Accepted date: 16-12-2014

Please cite this article as: Sarhan, W. A., and Azzazy, H. M. E.,High Concentration Honey Chitosan Electrospun Nanofibers: Biocompatibility and Antibacterial Effects, *Carbohydrate Polymers* (2015), http://dx.doi.org/10.1016/j.carbpol.2014.12.051

Highlights:

- Chitosan (5.5%) and honey (40%) were electrospun with poly vinyl alcohol to generate nanofibers.
- The nanofibers were characterized and studied with regard to swelling and weight loss.
- The developed nanofibres showed pronounced antibacterial activity against *S. aureus*.
- Tissue culture studies revealed biocompatibility of the nanofibrous scaffolds.

High Concentration Honey Chitosan Electrospun Nanofibers: Biocompatibility and Antibacterial Effects

Wessam A. Sarhan and Hassan M. E. Azzazy*

Novel Diagnostics & Therapeutics Research Group, Department of Chemistry, School of Sciences & Engineering, The American University in Cairo, New Cairo, Egypt 11835

Corresponding author:

Hassan M. E. Azzazy, PhD

Department of Chemistry

School of Sciences & Engineering, SSE # 1184

The American University In Cairo

AUC Avenue, P.O. Box 74

New Cairo, Egypt 11835

E-mail: hazzazy@aucegypt.edu

Fax: +2 02 2795 7565

Abstract

Honey nanofibers represent an attractive formulation with unique medicinal and wound healing advantages. Nanofibers with honey concentrations of □10% were prepared, however, there is a need to prepare nanofibers with higher honey concentrations to increase the antibacterial and wound healing effects. In this work, chitosan and honey (H) were cospun with polyvinyl alcohol (P) allowing the fabrication of nanofibers with high honey concentrations up to 40% and high chitosan concentrations up to 5.5% of the total weight of the fibers using biocompatible solvents (1% acetic acid). The fabricated nanofibers were further chemically crosslinked, by exposure to glutaraldehyde vapor and physically crosslinked by heating and freezing/thawing. The new HP-chitosan nanofibers showed pronounced antibacterial activity against *Staphylococcus aureus* but weak antibacterial activity against *Escherichia coli*. The developed HP-chitosan nanofibers revealed no cytotoxicity effects on cultured fibroblasts. In conclusion, biocompatible, antimicrobial crosslinked Honey/PVA/chitosan nanofibers were developed which hold potential as effective wound dressing.

Key words: Chitosan, honey, wound dressing, antibacterial, nanofibers.

Chemical compounds:
Chitosan: (PubChem CID: 71853); PVA (PubChem CID: 11199); Acetic acid (PubChem CID: 176); Glutaraldehyde (PubChem CID: 3485)

1. Introduction

Electrospinning is recognized as an efficient method for producing nanofibers (Li & Xia, 2004). The electrospun fibers show the advantages of high porosity and large surface to volume ratio (Altstädt, Lovera, Schmidt, Schmidt & Fery, 2008). Moreover, nanofibers resemble the natural extracellular matrix and was reported to promote proliferation and migration of cells (Bhardwaj & Kundu, 2010). Electrospun nanofibers represent an efficient formulation for drugs and natural remedies as they allow loading high concentration of combinations of natural and synthetic materials and controlled/sustained release (Meinel, Germershaus, Luhmann, Merkle & Meinel, 2012).

Honey has profound medicinal and nutritional properties (Khan, Abadin & Rauf., 2007). It exhibits antimicrobial activity, debriding and deodorising action as well as anti-inflammatory, antioxidant and wound healing activities (Lusby, Coombes, & Wilkinson, 2002). In 2013, Maleki et al. were able to fabricate honey/PVA nanofibers. Unfortunately, the maximum concentration that could be incorporated within the electrospun nanofibers was 2.25% honey of the total weight of the nanofibrous mat (Maleki, Gharehaghaji & Dijkstra, 2013). Recently, Wang and He (2013), worked on fabrication of high honey concentration nanofibers, however, the maximum concentration of included honey was 9% with 10% PVA of the total weight of the nanofibrous mat (Wang & He, 2013). Thus, there is a need to fabricate nanofibers composed primarily of high honey concentrations. Such concentrations will maximize the therapeutic and nutritional benefits of honey nanofibrous formulations in smaller dosage forms.

Chitosan is a biodegradable, biocompatible polymer with antibacterial, aqueous adsorption and wound healing ability (Schiffman & Schauer, 2007), in addition to its capacity to promote tissue regeneration and achieve hemostasis (Busilacchi, Gigante, Mattioli-Belmonte, Manzotti & Muzzarelli, 2013; Muzzarelli, Greco, Busilacchi, Sollazzo, & Gigante, 2012). Chitosan meets also the demands of several industrial and biomedical activities (Muzzarelli, 2010; Muzzarelli et al., 2012; Muzzarelli, El Mehtedi, Mattioli-Belmonte, 2014).

Because of the high viscosity of chitosan in solutions, electrospinning of chitosan was only possible by using toxic or highly concentrated acidic solvents (Geng, Kwon & Jang, 2005; Homayoni, Ravandi & Valizadeh, 2009; Su et al., 2011). Residues of such solvents are unfavourable especially in applications requiring biocompatible materials. Aqueous salts of chitosan were prepared, but the concentration of the incorporated chitosan did not exceed 1% (Charernsriwilaiwat, Opanasopit, Rojanarata, Ngawhirunpat, & Supaphol, 2010; Charernsriwilaiwat, Opanasopit, Rojanarata, & Ngawhirunpat, 2011). Another approach for electrospinning chitosan in more biocompatible solvents was via co-spinning with other readily spun polymers. Among

them, co-spinning chitosan with PVA is one of the most common composites (Liao et al., 2011; Yan et al., 2012; Zhou et al., 2007). Still, nanofibers prepared by this method could only incorporate limited chitosan concentrations.

It is the aim of the present work to co-spin high concentrations of chitosan and honey with PVA using biocompatible solvents. This would maximize the benefit of these two important materials in the smallest dosage form.

2. Experimental

2.1 Materials

Chitosan (Mwt: 240 kDa, DDA: 84%; Chitoclear, cg110, TM 3728; Primex; Siglufjordur, Iceland). PVA (Mwt: 85,000; Sigma Aldrich, St. Louis, USA), acetic acid (glacial, 99–100%; Merck, Wadeville, South Africa), glutaraldehyde (25% in H_2O; Sigma Aldrich, St. Louis, USA). Nutrient broth & Nutrient agar (Becton Dickinson and Company, USA). Trypsin (85450C-25G; Sigma Aldrich), RPMI_1640 with L-Glutamine (R8758; Life Science), Fetal Bovine serum (10270-106; Gibco), Thiazolyl Blue Tetrazolium Bromide – MTT (M2128-1G; Sigma Aldrich), PBS, trypan blue and triton X (Sigma Aldrich, St. Louis, USA). Clover honey was obtained from the faculty of Agriculture, Cairo University. The viscosity of the honey was 15300 mpas and its total soluble solid content was 81%.

2.2 Preparation of the chitosan/PVA(P-chitosan), honey/PVA (HP) and chitosan/honey/PVA (HP-chitosan) solutions

Different solutions composed of different weight ratios of P-chitosan and HP as well as HP-chitosan were prepared as follows: P-chitosan (7%:1.5%, 7%:2.5% and 7%:3.5%); HP (20%:10% and 30%:10%), and HP-chitosan (30%:7%:1.5%,30%:7%:3.5%, 30%:7%:5.5%, 20%:7%:3.5% and 40%:7%:3.5%). Solutions were prepared in 1% acetic acid. Each of the as prepared solutions of HP-chitosan was aged at room temperature for different time intervals.

2.3 Viscosity measurements

The viscosity of the PVA (7%), P-chitosan (7%:3.5%), HP (30%:7%), and HP-chitosan (30%:7%:3.5% and 10%:7%:3.5%) samples were determined using a viscometer (Myr; VR-3000, Viscotech Hispania, Tarragona, Spain). The solutions were aged at room temperature for a week. The viscosity of all samples was tested at different time intervals (0, 24, 48 h and 1 week). The average value of three measurements was reported as mean ± SD.

2.4 Electrospinning of chitosan/PVA (P-chitosan), honey/PVA (HP) and chitosan/honey/PVA (HP-chitosan) nanofibers

Each of the as-prepared solutions of P-chitosan, HP and HP-chitosan with different weight blending ratios was electrospun into nanofibers via the electrospinner (E-spin, NanoTech, Kalyanpur, India). The solutions were loaded in a 5 ml plastic syringe that was attached to a stainless steel needle (22 gauge) as a nozzle. The electrospun polymer solutions were subjected to different voltages (Gamma High Voltage power supply, USA) for adjustment of the optimum voltage for each of the spun solutions. The flow rate of the solution was maintained at 10 ul/min and the distance between the nozzle and the collector was maintained at 15 cm. Collection of the samples was done on a ground collector wrapped with an aluminium sheet.

2.5 Cross-linking of fibre mats

Physical and chemical methods were used to crosslink the fibre mats of HP-chitosan. Glutaraldehyde (GA) was used for chemical crosslinking. The fibre mats were placed in a closed desiccator that was saturated with GA vapors (40 ml). Exposure of the nanofiber mats to the GA vapors was done for different time intervals (30, 60, 120 and 180 min as well as 48 h and 72 h). Subsequently, enhancement of the crosslinking reaction and removal of unreacted (GA) was done via heating the nanofiber mats in an oven under vacuum at 70℃ for 24 h as well as at 40℃ for 24 h. Physical crosslinking was performed by freezing/thawing and heating techniques. Freezing and thawing was performed via freezing the fibre mats for 15 min in liquid nitrogen followed by thawing at room temperature for 15 min for three successive cycles. Heating was carried out under vacuum in an oven (Jeiotech, OV-11, South Korea) at both 110℃, 100 ℃ for 15 min and 80℃ for 25 min as well as at 70 ℃ for 24 h.

2.6 Characterization and measurements of the electrospun nanofibers

The morphologies of the electrospun nanofibers were observed using scanning electron microscopy (FESEM, Leo Supra 55, Zeiss Inc., Oberkochen, Germany). Fourier transform infrared spectroscopy (FTIR) was performed for the raw PVA and chitosan and the HP-chitosan nanofibrous mats using FTIR (Thermo scientific, Nicolet 380, USA). The transmission mode with KBr pellets was used for bulk chitosan and PVA as well as and HP-chitosan nanofibrous mats.

2.7 Degree of swelling and weight loss

The HP-chitosan nanofibrous mats were tested for the degree of swelling and weight loss that were calculated according to equations 1 and 2, respectively. Both tests were carried out in phosphate buffered saline [PBS], pH (7.4) at 37℃ for 1, 4 and 24 hours.

Degree of swelling (%) = [M-Mi/Mi] x 100 (equation 1) (Sharma, Dina & Mishra, 2013)
Weight loss (%) = [Mi-Md/Mi] x100 (equation 2)

Where M is the swollen weight of the nanofibrous sample which was dried using a filter paper, Md is the dried mass of the nanofibrous sample after being immersed in buffer medium, measured by drying the swollen mats at 40℃ u ntil constant weight was reached, and Mi is the initial dry mass of sample.

2.8 Assessment of antibacterial activity

Viable cell count technique was used to determine the antibacterial activity of the electrospun HP-chitosan nanofibrous mats with 30% honey/7%PVA and increasing chitosan concentrations [1.5%, 3.5%, and 5.5%]. The antibacterial activity was assessed against both *S. aureus* and *E. coli*. Each of the *S. aureus* and *E. coli* were added into 10mL nutrient broth medium that was adjusted to an OD of 0.1 at 625 nm. Subsequently, (0.1 g) of the HP-chitosan nanofibrous mats were added to each of the *S. aureus* and *E. coli* test tubes. All the nanofibrous mats were UV sterilized for 20 min prior to antibacterial testing. The *S. aureus* and *E. coli* tubes containing the nanofibrous mats and a control were then incubated at 37℃ with shaking at 100 rpm. Samples from the treated bacterial broth and the control were taken and serially diluted in nutrient broth at 24 and 48 h. Subsequently, 100 uL from each dilution were spread on nutrient agar plates that were then incubated at 37℃ for 24 h , after which the numbers of surviving colonies were counted.

The antibacterial activity was estimated according to equation 3:
Antibacterial activity= (log CFU* t- log CFU*0) – (log CFUt- log CFU0) (equation 3)

Where CFU0 and CFUt are the number of colony forming units at time zero and time t for the nanofibrous samples; CFU*0 and CFU*t are the number of colony forming units at time zero and time t for the control (Amrit, Hendrix, Dutschik & Warmoeskerken, 2012).

2.9 Cytotoxicity evaluation (MTT assay)

Primary skin fibroblast cells of neonatal mice origin were used to evaluate the toxicity of the HP-chitosan nanofibrous mats with increasing chitosan concentrations [1.5%, 3.5%, and 5.5%] and 30% honey/7%PVA. Preparation of the primary cell culture was done according to the method of Seluanov, et al., 2010 (Seluanov, Vaidya, & Gorbunova, 2010). Non crosslinked and crosslinked nanofibrous mats were tested for each

concentration. Crosslinking was achieved via exposure to GA vapors for 180 min, followed by heating at 70℃ under vacuum. Cytotoxicit y was evaluated via the addition of the extracts of the nanofiber scaffolds to cells cultured in a 24-well plate and the cytotoxicity was determined via MTT assay. The nanofibrous mats were extracted via soaking the scaffolds in culture media for 24 h at 37℃. Subsequently the extracts were harvested for cytotoxicity testing. Normal cells without any treatment were used as the negative control whereas (1% Triton X) was used as the positive control. The cells were seeded in a 24 well plate at a density of 10^4 cells/well and incubated in a humidified incubator with 5% CO_2 for 24h at 37℃ before treatment with the extracts t o allow cell attachment. Subsequently, the extract for each scaffold was added to the cell monolayer and incubated for 48 h into CO_2 incubator at 37 ℃ and 5% CO_2. Triplicate wells were prepared for each sample. After 48 h, the difference in morphology between cell controls and scaffold extracts was observed by observing the cells under inverted microscope. Cell viability was assessed after 3 days via MTT assay. The absorbance was determined at 570 nm. And percent of cell survival was calculated according to the following equation:

Survival %= $[A_{sample} - A_b / A_c - A_b] \times 100$ (equation 4)

A_c is the negative control

A_b is the blank

The average value of three measurements was reported as mean ± SD. Analysis of the data was done via analyses of variance (ANOVA) test. And results were considered statistically significant with a probability less than 0.05.

2 Results and discussion

3.1Preparation of chitosan/PVA, honey/PVA and chitosan/ honey/PVA nanofibers

The solutions of P-chitosan, HP, and HP-chitosan were tested for viscosity at different time intervals as shown in table 1. At zero time, the viscosity of (HP; 30%:7%) was very low (175 mpas) and the viscosity of the (P-chitosan; 7%:3.5%) was very high (85440 mpas) making both solutions impossible to spin. Whereas, the combination of (HP-chitosan; 30%:7%:3.5%) exhibited 34000 mpas at day zero. Such viscosity value, however was still above the optimum viscosity required for spinning. Thus, the HP-chitosan solutions were allowed to age at room temperature for a week. Interestingly, the viscosity of the HP-chitosan solutions dropped noticeably upon aging. This was unlike the P-chitosan and the HP solutions that exhibited increased viscosities after aging for one week [Table 1].

>>>Inert Table 1

The decrease in viscosity of the HP-chitosan solutions with time could be due to enzymatic degradation of chitosan via the enzymes present in the honey. Small amounts of enzymes occur naturally in honey, including enzymes that transform polysaccharides into smaller products as amylase. Chitosan is most likely to be affected by such enzymes (Xie, Jia, Huang & Zhang, 2011). Moreover, hydrogen peroxide which is an important component of honey may have contributed to the enzymatic degradation of chitosan (Brudzynski, 2006). Interestingly, it was observed that the increase in the honey concentration within the HP-chitosan mixtures has resulted in further reduction in the viscosity of the solutions [Table 1].

3.2 Morphology of the chitosan/PVA, honey/PVA and chitosan/ honey/PVA nanofibers

Different concentrations of the P-chitosan, HP and HP-chitosan were electrospun. For P-chitosan combinations, the highest concentration of chitosan that could be electrospun with PVA using 1% acetic acid, was 1.5%. For HP combinations the highest concentration of honey that could be electrospun with PVA was 20% honey [fig. 1a]. However the electrospun fibers showed clusters, which are most probably clusters of honey which were not included within the PVA nanofibers. Remarkably, upon addition of 3.5% chitosan to the same HP combination, uniform nanofibers were produced [fig 1b]. This is due to the favourable effect of chitosan on the viscosity of the solution allowing it to reach to the optimum degree of chain entanglements required to form uniform nanofibers.

>>> Insert Figure 1

Upon increasing the honey concentration to 30% in the HP combination the honey clusters increased extensively [fig. 1c] indicating the inability of the PVA polymer to incorporate higher concentrations of honey even at higher concentrations of PVA, where the decrease in viscosity imparted by honey on the HP combination could not be overcome by increasing the concentration of PVA. On the other hand, increasing the chitosan concentration to 3.5% in the P-chitosan resulted in highly viscous solution that was impossible to spin [Table 1]. Interestingly, the combination HP-chitosan (30%:7%:3.5%), upon aging for more than 2 days acquired the optimum viscosity required for easy spinning and formation of uniform nanofibers [fig. 1d]. Such combination of HP-chitosan allowed for the first time the production of biocompatible fibers via biocompatible solvents of high concentrations of both honey and chitosan.

Realizing the synergistic effect of both honey and chitosan on the viscosity of the HP-chitosan combinations, attempts were made to increase the concentration of the incorporated honey and chitosan. Spinning 35% and 40% honey within the combination of chitosan (3.5%)/ PVA (7%) was successful [figures 2a & 2b]. Also, spinning 4.5% and

5.5% chitosan in the presence of 30% honey was achieved [figures 2c & 2d]. However due to the high viscosity of the increased concentration of chitosan the concentration of PVA incorporated in such a combination was decreased to 5%.

>>> Insert Figure 2

In previous attempts to prepare nanofibers containing high honey concentration, the maximum incorporated concentration that was electrospun with PVA was 9% (Wang & He, 2013). This is because increasing the honey concentration results in remarkable decrease in viscosity of the solution, thus making it impossible to electropsin. Remarkably, this is the first report to prepare nanofibers with honey concentrations reaching to 40% of the actual weight of the nanofiberous mat. Furthermore, the favourable effect of honey on the viscosity of the chitosan solution upon aging allowed for the first time for incorporating higher chitosan concentrations reaching to 5.5% while using biocompatible solvents.

The FTIR analysis of the PVA, CH and HP-chitosan nanofibers was carried out and analysed. Chitosan exhibited characteristic bands at 3429 $cm^{-1}$and 1655 cm^{-1} corresponding to the OH and the amide O-C-NH2 groups. The bands of the CH3 and CH3-O groups could be observed between 1000-2000 cm^{-1} (Paipitak, Pornpra, Mongkontalang, Techitdheer & Pecharapa, 2011). The FT-IR spectra of PVA showed bands at 3429 cm^{-1}, 2923 cm^{-1}, and 1444 cm^{-1} the characteristic bands for OH, CH2, and CH-OH groups (Yan et al., 2012). The previous characteristic bands of both PVA and chitosan were all preserved in the resulting hybrid fibers. However, it was observed that the absorption peak at about 3429 cm^{-1} and 1655 cm^{-1} concerned with OH and amide O-C-NH2 groups shifted to a lower wave number in the composite HP-chitosan. At the same time, the characteristic peak in the hybrid HP-chitosan at 1058 cm^{-1} could be attributed to the C-O-C symmetric stretching and C-O-H bending vibrations of protein in honey. Whereas, the amide band of protein in honey could be observed at 1641 cm^{-1} (Philip, 2009). Moreover, the peaks between 900 cm^{-1} and 750 cm^{-1} were attributed to the anomeric region, which is a characteristic of saccharide configuration of honey (Jaganathan & Mandal, 2009; Philip, 2010).

3.3. Morphology before and after cross-linking treatment

It was observed that the nanofibrous scaffolds of HP-chitosan combinations lose their nanofibrous structure in aqueous media. Thus, efficient crosslinking was necessary to broaden the possible applications of the developed nanofibers.

Through the present work different crosslinking strategies were undertaken, to allow efficient crosslinking without jeopardizing the biocompatibility of the fibers. In chemical crosslinking the temperature of heating did not exceed 110 ℃. This is because excessive heating above 140℃ can result in reduction of the honey quality and increase in the hydroxymethylfurfural content (Tosi, Ré, Lucero & Bulacio, 2004). Figure 3 shows the images of the chemically cross-linked nanofibers after immersion in PBS for 15 min.

>>> Insert Figure 3

The fibers that were subjected to GA vapors for three days showed superior crosslinking [fig. 3a] and maintained their original shapes and no swelling was observed. Fibers subjected to GA vapors for 2 days showed similar results however slight swelling was observed [fig. 3b]. Interestingly, decreasing the exposure time to GA vapors to 3 h maintained their nanofibrous structure with some swelling [fig. 3c]. Meanwhile, decreasing the exposure time to 1 h [fig. 3d] showed lower crosslinking efficiency, where partial degradation of the outer layers of the fibers began with noticeable swelling. However, crosslinking efficiency decreased noticeably upon decreasing the GA exposure time to 30 min, where the percentage of the degraded fibers increased and the nanofibrous structure in the outer layer was nearly lost. Subjecting the nanofibers to GA vapors for 1 h and 3h, with subsequent heating for 24 h to promote crosslinking at 40℃, showed the same crosslinkin g efficiency as nanofibers heated at 70℃. It was reported that exposing honey t o 40^{o}C for 96 h did not affect any of its biomolecules (Molan, 1992).

Among the different physical crosslinking procedures applied, cross-linked fibers could only be achieved by heating at 110 ℃ for 15 min [fi g. 4a], it could be observed also that such fibers exhibited noticeable swelling. Meanwhile, heating at 70℃ for 24 h showed partially degraded swollen fibers [fig. 4b]. Heating induces the crystallization of the incorporated polymers (Kang et al., 2010). Freezing and thawing in liquid nitrogen as well as heating at elevated temperatures made the nanofibrous scaffold brittle and liable to cracking.

>>> Insert Figure 4

It is worth noting, that upon physical cross-linking by heating, a change in the color of the nanofibers was observed from white to light brown. The same effect was observed upon aging of the nanofibers for several months. Such color change may indicate possible interactions between the sugar aldehyde groups and the chitosan amino groups.

3.4 Weight loss and water retention behaviour

The water uptake capability and degree of weight loss of the electrospun fibers were investigated. As shown in figure 5a, the noncrosslinked fibers exhibited swelling capabilities between 46% to 197%, with the highest swelling observed for the sample containing 3.5% chitosan and 20% honey (HP-chitosan: 20%:7%:3.5%) tested at 4 h. Although, PVA, chitosan and honey enhance water uptake, the samples showed moderate swelling capabilities when compared to previously spun chitosan and PVA fibers lacking honey. Jannesari et al. (2011), reported that the swelling value of PVA/chitosan nanofibers was 390% after 24h compared to 135% for the HP-chitosan: 3.5%:20%:7% in the present work.

>>> Insert Figure 5

Such results may be attributed to the high water solubility of honey, where although honey increases the water uptake (MohdZohdi, Abu BakarZakaria, Yusof, Mohamed Mustapha & Abdullah, 2011), its high water solubility leads to an increase in the degradation rate of the fibers. Similarly, Wang and co-workers observed the same effect upon inclusion of 20% honey in a gelatine/chitosan/honey hydrogel. Honey first promotes swelling due to its high osmolarity, however, upon water uptake the high water solubility of honey accelerates the degradation rate and thus results in low swelling due to the absence of a compact structure to retain the water (Wang, Zhu, Xue & Wu, 2012). Thus, the highest swelling percent in all tested samples were observed at 4 h that decreased at 24 h. Moreover, upon comparing the HP-chitosan samples of 30%:7%:3.5% and 20%:7%:3.5% [fig. 5a], it was observed that an increase in the water uptake capability is achieved upon decreasing the honey concentration.

On the other hand, chitosan with its decreased water solubility decreases the weight loss within the HP-chitosan nanofibrous mats, which is observed upon comparing the decrease in swelling and the increase of the weight loss of the (HP-chitosan; 30%:7%:1.5%) compared to (HP-chitosan; 30%:7%:3.5%) [figs. 5a & 5b]. However, upon increasing the chitosan concentration to 5.5% in the HP-chitosan nanofibers, a marked increase in the swelling percent was only observed at 24 h. This is because, although chitosan enhances water uptake, increasing the chitosan concentration above a certain level does produce the opposite effect. This was explained by Son and co-workers, who observed that in the chitosan/PVA nanofibrous scaffolds of low chitosan concentrations, the hydrophilic PVA could easily form polymeric hydrogels in solutions thus allowing enhanced swelling. Whereas, above certain concentration of chitosan, the intermolecular forces between the chitosan side chains and the amine groups increase, thus leading to decreased swelling (Son, Yeom, Song, Lee & Hwang, 2009).

3.5Antibacterial evaluation

The antimicrobial activity of honey is due to its ability to produce hydrogen peroxide, its high sugar content, its acidity and its content of flavonoids (Vandamme et al., 2013). On the other hand, the antibacterial activity of chitosan is mainly due to the interaction between the chitosan polycations and the negatively charged surfaces of bacteria, which leads to loss of bacterial membrane permeability leading to cell leakage and death (Muzzarelli, Tarsi, Filippini, Giovanetti, Biagini, & Varaldo, 1990).

>>> Insert Figure 6

Considering its biodegradable nature, the antibacterial activity of the HP-chitosan nanofibrous mats is dependent on the concentration of its components in the media which increases with time. As shown in figure 6a, the antibacterial activity against *S.aureus* increased with increasing the chitosan concentration within the HP-chitosan nanofibers. Moreover, increasing the incubation time resulted in marked increase in antibacterial activity especially with the 3.5% and 5.5% incorporated chitosan concentrations. Complete bacterial inhibition was achieved at 48 h with the 5.5% chitosan. This may be attributed to the decreased solubility of chitosan, thus at longer incubation periods larger percentage of chitosan is degraded thus leading to increased antibacterial activity.

Testing the HP-chitosan nanofibrous mats on *E. coli* revealed weak antibacterial activity [fig. 6b]. Such results agree with the results of No et al. (2002) who observed the weak antibacterial activity of chitosan against gram negative bacteria (No, Young Park, Ho Lee, & Meyers, 2002).

It is worth mentioning that the nanofibrous structure enhanced the antibacterial activity of the included components. The tested sample (0.1 g) contains less than 20 ppm chitosan and approximately 0.175% honey and produced pronounced antibacterial effects against *S.aureus* and weak antibacterial effects against *E. coli* compared to no antibacterial effect at such concentrations for both honey and chitosan alone (Goy, Britto & Assis, 2009; Islam,Masum, Mahbub & Haque, 2011; Liu et al, 2006; Mandal & Mandal, 2011). Such results could be attributed to the dramatic increase in the surface to volume ratio of the nanofibers.

3.6 Cytotoxicity evaluation

Cells grown with the extracts of the HP-chitosan nanofibers showed similar morphologies to that of the negative control (data not shown). Primary skin fibroblast cells were cultured with the extract of the crosslinked and noncrosslinked HP-chitosan nanofibrous scaffolds having the concentrations of 30%:7%:1.5%, 30%:7%:3.5% and 30%:7%:5.5% for 3 days and their toxicities were evaluated using MTT assay as shown in Figure 7.

>>> Insert Figure 7

Cells cultured with the HP-chitosan nanofibrous scaffolds exhibited no significant differences in cell viability to those of the negative control (cultured with no nanofibrous scaffolds) (p□ 0.05) and significantly different and improved viability than the cells cultured with the positive control. Such results indicate the biocompatibility of the developed HP-chitosan nanofibrous scaffolds. It was realized however, that the crosslinked nanofibers in all tested nanofibrous samples showed slight decrease in the viability of the cells compared to the noncrosslinked nanofibers. This may be due to the traces of GA remaining on the nanofibers. Still, the viability of the cells cultured with the crosslinked nanofibers after 3 days were similar to those of the negative control indicating good biocompatibility.

3 Conclusions

In this study, PVA was co-spun with honey and chitosan resulting in HP-chitosan nanofibers with honey concentrations ranging from 20% to 40% and chitosan concentrations ranging from 1.5% to 5.5%. The combination of chitosan and honey had a synergistic effect on the viscosity of the solution allowing it to reach the optimum viscosity required for electrospinning. Such effect allowed for the first time for fabrication of nanofibers comprising 40% of their actual weight honey compared to 9% in previous attempts and up to 5.5% chitosan without the use of high concentrated acids or toxic solvents. Physical and chemical crosslinking of the developed HP-chitosan nanofibers resulted in different degrees of crosslinking which may extend their applications. The developed nanofibers (HP-chitosan; 30%:7%:3.5%) exhibited enhanced antibacterial activity against *S. aureus* but poor antibacterial activity against *E. coli*. The antibacterial activity increased by increasing the concentration of the incorporated chitosan within the nanofibers from 1.5% to 5.5%. Additionally, changing the concentrations of chitosan and honey resulted in different degrees of water uptake ranging from 46% to 197%. The degradation rate of the developed nanofibers was inversely related to the concentration of the chitosan within the nanofibers. Glutaraldehyde crosslinked and noncrosslinked

nanofibers had no toxicity on cultured primary fibroblasts. The developed HP-chitosan nanofibers with high concentrations of honey and chitosan hold the potential as effective biocompatible wound dressings.

4 Acknowledgements

The authors would like to thank Dr. Mahmoud El. Syed Nour, Faculty of Agriculture, Cairo University, Giza, Egypt for his generous supply of the clover honey and its specifications.

5 References

Altstädt, V., Lovera, D., Schmidt, H., Schmidt, S., & Fery, A. (2008). Electrospun Polymeric Fine Fibers. *Imaging & Microscopy*, *10* (2), 29-31.

Amrit, U.R, Hendrix, R., Dutschk, V &Warmoeskerken M.M.C.G & (2012). *A Study of the Antibacterial Activity of PolyhexamethyleneBigaunide on Cotton Substrate.* In: 12th AUTEX World Textile Conference - Innovative textile for high future demands, 13th to 15th June 2012, 13 June 2012, Zadar, Croatia

Bhardwaj, N., & Kundu, S. C. (2010). Electrospinning: a fascinating fiber fabrication technique. *Biotechnology advances*, *28*(3), 325-347.

Brudzynski, K. (2006). Effect of hydrogen peroxide on antibacterial activities of Canadian honeys. Canadian Journal of Microbiology, 52(12), 1228-1237.

Busilacchi, A., Gigante, A., Mattioli-Belmonte, M., Manzotti, S., Muzzarelli, R. A. A., (2013). Chitosan stabilizes platelet growth factors and modulates stem cell differentiation toward tissue regeneration. *Carbohydrate Polymers*, 98 (1):665-676.

Charernsriwilaiwat, N., Opanasopit, P., Rojanarata, T., Ngawhirunpat, T., & Supaphol, P. (2010). Preparation and characterization of chitosan-hydroxybenzotriazole/polyvinyl alcohol blend nanofibersby the electrospinning technique. *Carbohydrate Polymers*, *81*(3), 675-680.

Charernsriwilaiwat, N., Opanasopit, P., Rojanarata, T., &Ngawhirunpat, T. (2011). Fabrication and characterization of chitosan-ethylenediaminetetraacetic acid/polyvinyl alcohol blend electrospunnanofibers. *Advanced Materials Research*, *194*, 648-651.

Geng, X., Kwon, O. H., & Jang, J. (2005). Electrospinning of chitosan dissolved in concentrated acetic acid solution. *Biomaterials*, *26*(27), 5427-5432.

Goy, R. C., Britto, D. D., & Assis, O. B. (2009). A review of the antimicrobial activity of chitosan. *Polímeros*, 19(3), 241-247.

Homayoni, H., Ravandi, S. A. H., & Valizadeh, M. (2009). Electrospinning of chitosan nanofibers: Processing optimization. *Carbohydrate Polymers*, *77*(3), 656-661.

Islam, M., Masum, S. M., Mahbub, K. R., & Haque, M. (2011). Antibacterial Activity of Crab-Chitosan against Staphylococcus aureus and Escherichia coli.Journal of *Advanced Scientific Research*, 2(4).

Jaganathan, S. K., & Mandal, M. (2009). Antiproliferative effects of honey and of its polyphenols: a review. *Journal of Biomedicine and Biotechnology*, *2009*.

Jannesari, M., Varshosaz, J., Morshed, M., &Zamani, M. (2011). Composite poly (vinyl alcohol)/poly (vinyl acetate) electrospunnanofibrous mats as a novel wound dressing matrix for controlled release of drugs. *International Journal of Nanomedicine*, 6, 993-1003.

Kang, Y. O., Yoon, I. S., Lee, S. Y., Kim, D. D., Lee, S. J., Park, W. H., & Hudson, S. M. (2010). Chitosan-coated poly (vinyl alcohol) nanofibers for wound dressings. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, *92*(2), 568-576.

Khan, F. R., Abadin, Z. U., & Rauf, N. (2007). Honey: nutritional and medicinal value. *International journal of clinical practice*, *61*(10), 1705-1707.

Li, D., & Xia, Y. (2004). Electrospinning of nanofibers: reinventing the wheel? *Advanced materials*, *16* (14), 1151-1170.

Liao, H., Qi, R., Shen, M., Cao, X., Guo, R., Zhang, Y., et al. (2011). Improved cellular response on multiwalled carbon nanotube-incorporated electrospun polyvinyl alcohol/chitosan nanofibrous scaffolds. *Colloids and Surfaces B: Biointerfaces*, *84*(2), 528-535.

Liu, N., Chen, X. G., Park, H. J., Liu, C. G., Liu, C. S., Meng, X. H., & Yu, L. J. (2006). Effect of MW and concentration of chitosan on antibacterial activity of *Escherichia coli*. *Carbohydrate polymers*, 64(1), 60-65.

Lusby, P. E., Coombes, A., & Wilkinson, J. M. (2002). Honey: a potent agent for wound healing?. *Journal of Wound Ostomy & Continence Nursing*, 29(6), 295-300.

Maleki, H., Gharehaghaji, A. A., & Dijkstra, P. J. (2013). A novel honey-based nanofibrous scaffold for wound dressing application. *Journal of Applied Polymer Science*, *127*(5), 4086-4092.

Mandal, M. D., & Mandal, S. (2011). Honey: its medicinal property and antibacterial activity. Asian Pacific journal of tropical biomedicine, 1(2), 154-160.

Meinel, A. J., Germershaus, O., Luhmann, T., Merkle, H. P., &Meinel, L. (2012). Electrospun matrices for localized drug delivery: current technologies and selected biomedical applications. *European Journal of Pharmaceutics and Biopharmaceutics*, *81*(1), 1-13.

MohdZohdi, R., Abu BakarZakaria, Z., Yusof, N., Mohamed Mustapha, N., & Abdullah, M. N. H. (2011). Gelam (Melaleuca spp.) honey-based hydrogel as burn wound dressing. *Evidence-Based Complementary and Alternative Medicine*, 2012.

Molan, P.C. (1992). The antibacterial activity of honey. 2: Variation in the potency of the antibacterial activity. Bee World 73 (2), 59-76.

Muzzarelli, R.A.A., Tarsi, R., Filippini, O., Giovanetti, E., Biagini, G., & Varaldo, P.E. (1990). Antimicrobial properties of N-carboxybutyl chitosan. Antimicrobial Agents and Chemotherapy, 34, 2019-2023

Muzzarelli, R.A.A. (2010). Chitins and chitosans as immune adjuvants and non-allergenic drug carriers. *Marine Drugs*, 8(2), 292-312.

Muzzarelli, R.A.A., Boudrant J., Meyer D., Manno N., DeMarchis M., & Paoletti, M.G. (2012). Current views on fungal chitin/chitosan, human chitinases, food preservation, glucans, pectins and inulin: A tribute to Henri Braconnot, precursor of the carbohydrate polymers science, on the chitin bicentennial. *Carbohydrate Polymers*, 87, 995-1012.

Muzzarelli, R.A.A., Greco, F., Busilacchi, A., Sollazzo, V., & Gigante, A. (2012). Chitosan, hyaluronan and chondroitin sulfate in tissue engineering for cartilage regeneration: a review. *Carbohydrate Polymers*, 89, 723-739.

Muzzarelli, R.A.A., El Mehtedi, M., Mattioli-Belmonte, M. (2014). Emerging biomedical applications of nano-chitins and nano-chitosans obtained via advanced eco-friendly technologies from marine resources. *Marine Drugs*, 12, 5468-5502

No, H. K., Young Park, N., Ho Lee, S., & Meyers, S. P. (2002). Antibacterial activity of chitosans and chitosan oligomers with different molecular weights.*International journal of food microbiology*, *74*(1), 65-72.

Paipitak, K., Pornpra, T., Mongkontalang, P., Techitdheer, W., &Pecharapa, W. (2011). Characterization of PVA-chitosan nanofibers prepared by electrospinning. *Procedia Engineering*, *8*, 101-105.

Philip, D. (2009). Honey mediated green synthesis of gold nanoparticles. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, *73*(4), 650-653.

Philip, D. (2010). Honey mediated green synthesis of silver nanoparticles. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, *75*(3), 1078-1081.

Schiffman, J. D., & Schauer, C. L. (2007). Cross-linking chitosan nanofibers. *Biomacromolecules*, *8*(2), 594-601.

Seluanov, A., Vaidya, A., & Gorbunova, V. (2010). Establishing primary adult fibroblast cultures from rodents. Journal of visualized experiments: *Journal of visualized experiments*, (44).

Sharma, C., Dinda, A. K. & Mishra, N. C (2013). Fabrication and characterization of natural origin chitosan- gelatin-alginate composite scaffold by foaming method without using surfactant. *Journal of Applied Polymer Science*, *127*, 3228–3241.

Son, B., Yeom, B. Y., Song, S. H., Lee, C. S., & Hwang, T. S. (2009). Antibacterial electrospun chitosan/poly (vinyl alcohol) nanofiberscontaining silver nitrate and titanium dioxide. *Journal of Applied Polymer Science*, 111(6), 2892-2899.

Su, P., Wang, C., Yang, X., Chen, X., Gao, C., Feng et al. (2011). Electrospinning of chitosan nanofibers: The favorable effect of metal ions. *Carbohydrate Polymers*, *84*(1), 239-246.

Tosi, E. A., Ré, E., Lucero, H., & Bulacio, L. (2004). Effect of honey high-temperature short-time heating on parameters related to quality, crystallisation phenomena and fungal inhibition. *LWT-Food Science and Technology*, *37*(6), 669-678.

Vandamme, L., Heyneman, A., Hoeksema, H., Verbelen, J., &Monstrey, S. (2013). Honey in modern wound care: A systematic review. *Burns*. 39(8), 1514-1525.

Wang, P., & He, J. H. (2013). Electrospun polyvinyl alcohol-honey nanofibers.*Thermal Science*, *17*(5), 1549-1550.

Wang, T., Zhu, X. K., Xue, X. T., & Wu, D. Y. (2012). Hydrogel sheets of chitosan, honey and gelatin as burn wound dressings. *Carbohydrate Polymers*,88 (1), 75-83.

Xie, H., Jia, Z., Huang, J., & Zhang, C. (2011). Preparation of low molecular weight chitosan by complex enzymes hydrolysis. *International Journal of Chemistry*, *3*(2), p180.

Yan, E., Fan, S., Li, X., Wang, C., Sun, Z., Ni, L., & Zhang, D. (2012). Electrospun polyvinyl alcohol/chitosan composite nanofibersinvolving Au nanoparticles and their in vitro release properties. *Materials Science and Engineering: C*, *33*, 461–465.

Zhou, Y., Yang, D., Chen, X., Xu, Q., Lu, F., & Nie, J. (2007). Electrospun water-soluble carboxyethyl chitosan/poly (vinyl alcohol) nanofibrous membrane as potential wound dressing for skin regeneration. *Biomacromolecules*, *9*(1), 349-354.

633
634

Figure Legends

Figure 1. SEM images of the electrospun nanofibre mats with the highest concentration (%) of honey within the honey/polyvinyl alcohol (HP) and the HP-chitosan nanofibers: (a) HP (20%:8%) (b) HP-chitosan (20%:8%:3.5%) (c) HP (30%:7%) (d) HP-chitosan (30%:7%:3.5%).

Figure 2. SEM images of the electrospun nanofibre mats with the maximum concentration (%) of both honey and chitosan within the honey/polyvinyl alcohol/chitosan (HP-chitosan) nanofibers: (a) HP-chitosan (35%:7%:3.5%) (b) HP-chitosan (40%:7%:3.5%) (c) HP-chitosan (30%:7%:4.5%) (d) HP-chitosan (30%:7%:5.5%)

Figure 3. SEM images of the chemically cross-linked Honey/polyvinyl alcohol/chitosan (HP-chitosan) (30%:7%:3.5%) nanofibrous mats. Cross-linking was performed by exposure to GA vapors and then heating at 70℃ under vacuum for 24 h. Different mats were exposed to GA for different time intervals (a) 3 days (b) 2 days (c) 3 h (d) 1 h.

Figure 4. SEM images of the honey/polyvinyl alcohol/chitosan (HP-chitosan) (30%:7%:3.5%) nanofibre mats that exhibited physical cross-linking by: (a) heating at 110℃ for 15 min under vacuum, and (b) heating at 70 ℃ for 24 h under vacuum.

Figure 5. % Swelling [a] and % weight loss [b] of the honey/polyvinyl alcohol/chitosan (HP-chitosan) nanofiber mats with different weight ratios of HP-chitosan after immersion in PBS (pH 7.4) for 1, 4, and 24 h. Different weight ratios of the tested HP-chitosan included: (A) 30%:7%:1.5%, (B) 30%:7%:3.5%, (C) 30%:7%:5.5%, and (D) 20%: 7%:3.5%.

Figure 6. The antibacterial activity of the electrospun honey/polyvinyl alcohol/chitosan (HP-chitosan) of mats against *S. aureus* [a] and *E.coli* [b] at 24 and 48 h on 7 × 10^8 CFU/mL bacteria. The weight blending ratios of the electrospun mats were 7% polyvinyl alcohol, 30% honey and increasing concentrations of chitosan; (A) 1.5%, (B) 3.5%, and (C) 5.5%.

672 **Figure 7.** Effect of electrospun honey/polyvinyl alcohol/chitosan (HP-chitosan)
673 nanofibers on cultured fibroblasts investigated using MTT assay. Different HP-
674 chitosan nanofibrous scaffolds with 30% honey, 7% polyvinyl alcohol, and different
675 concentrations of chitosan: (A) 5.5%, (B) 3.5% and (C) 1.5%, were tested. Both non-
676 crosslinked and crosslinked HP-chitosan nanofibrous scaffolds were tested. The data
677 represents the mean ±SD (N= 3).

678

679 **Tables:**

680

681 Table 1. Change in the viscosity (mpas) of the polyvinyl alcohol (P), honey/P (HP), P-
682 chitosan, and HP-chitosan solutions upon aging.
683

Time`	P (7%) (mpas)	HP (30%:7%) (mpas)	P-chitosan (7%:3.5%) (mpas)	HP-chitosan (10%:7%:3.5%) (mpas)	HP-chitosan (30%:7%:3.5%) (mpas)
0 h	300	175	85440	48010	34000
24 h	328	214	162830	9770	6520
48 h	285	245	152020	6100	3830
168 h	404	319	122180	2787	1851

684

Figure 1

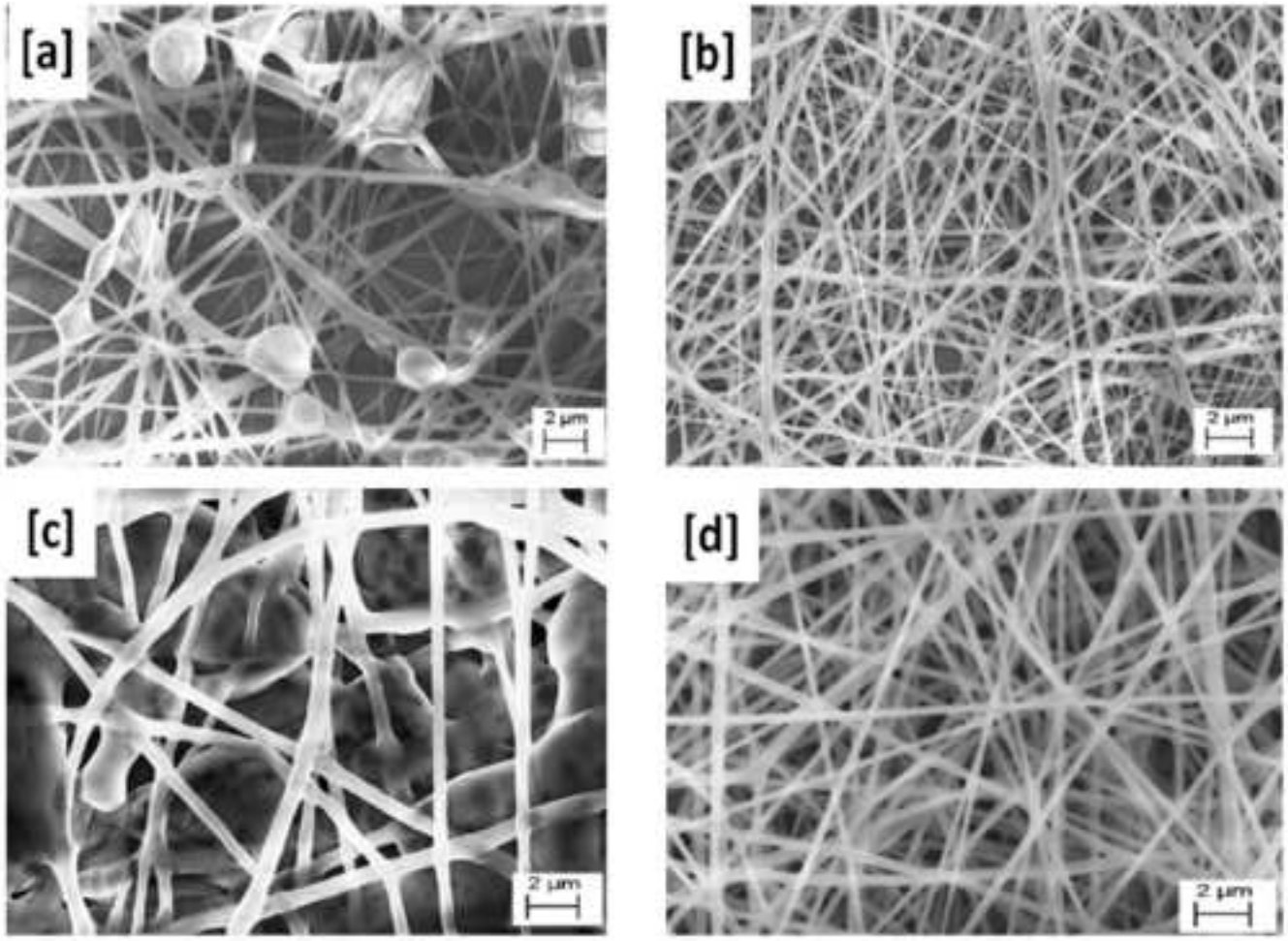


Figure 2

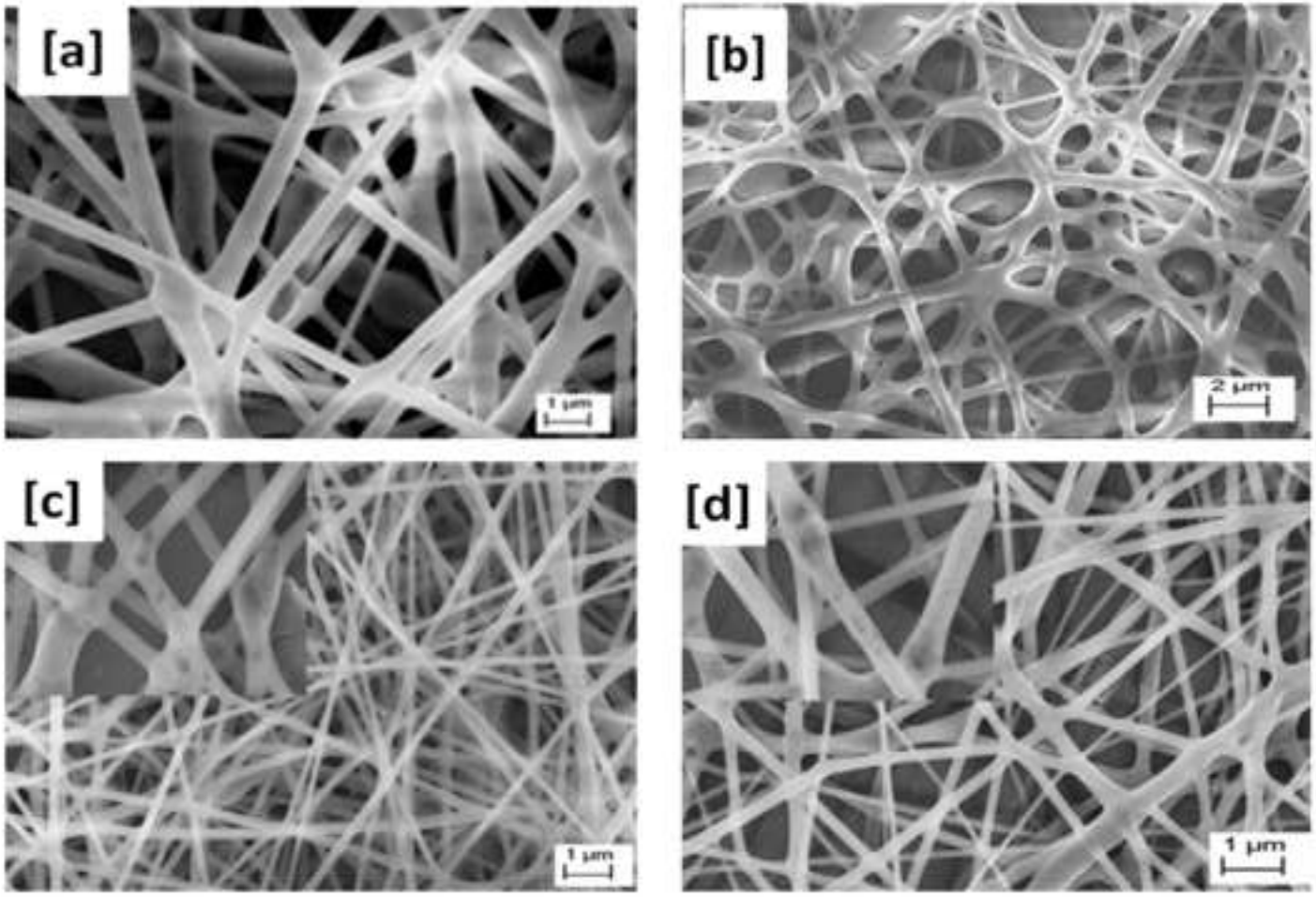


Figure 3

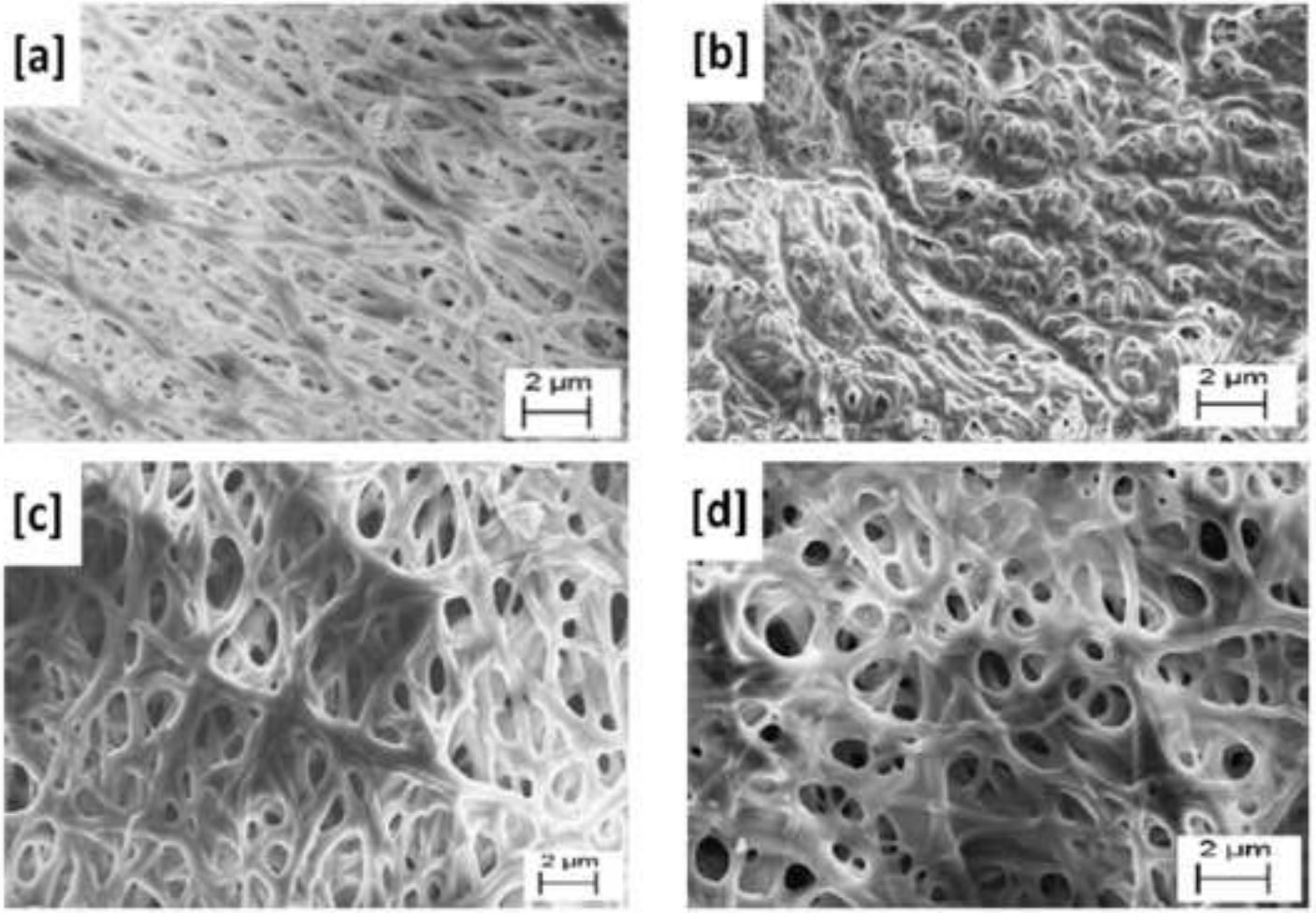


Figure 4

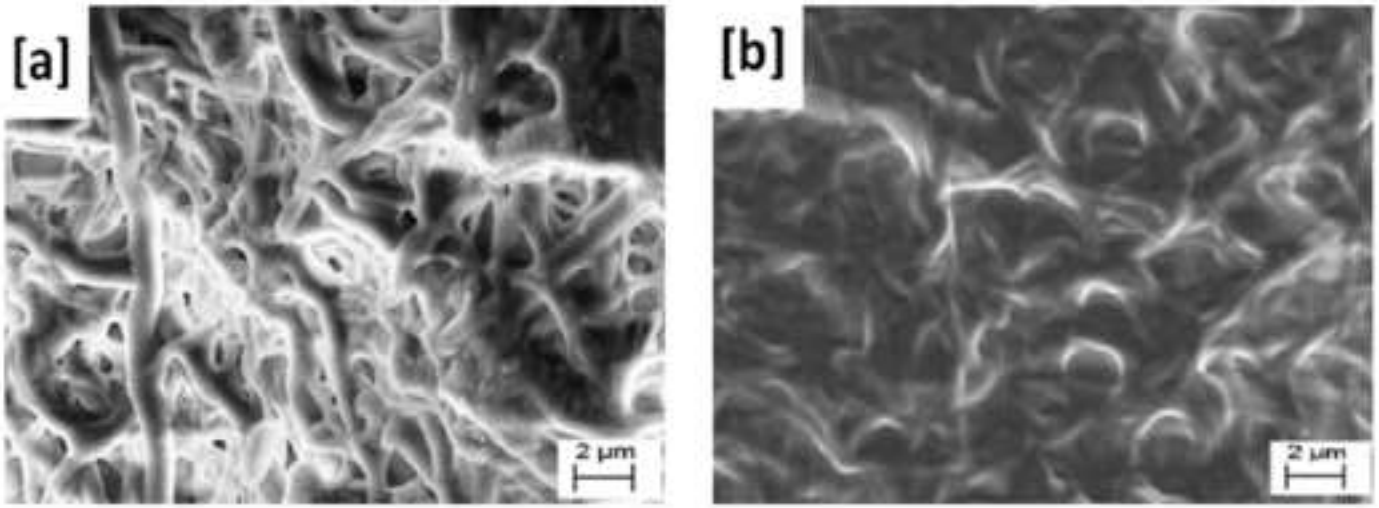


Figure 5

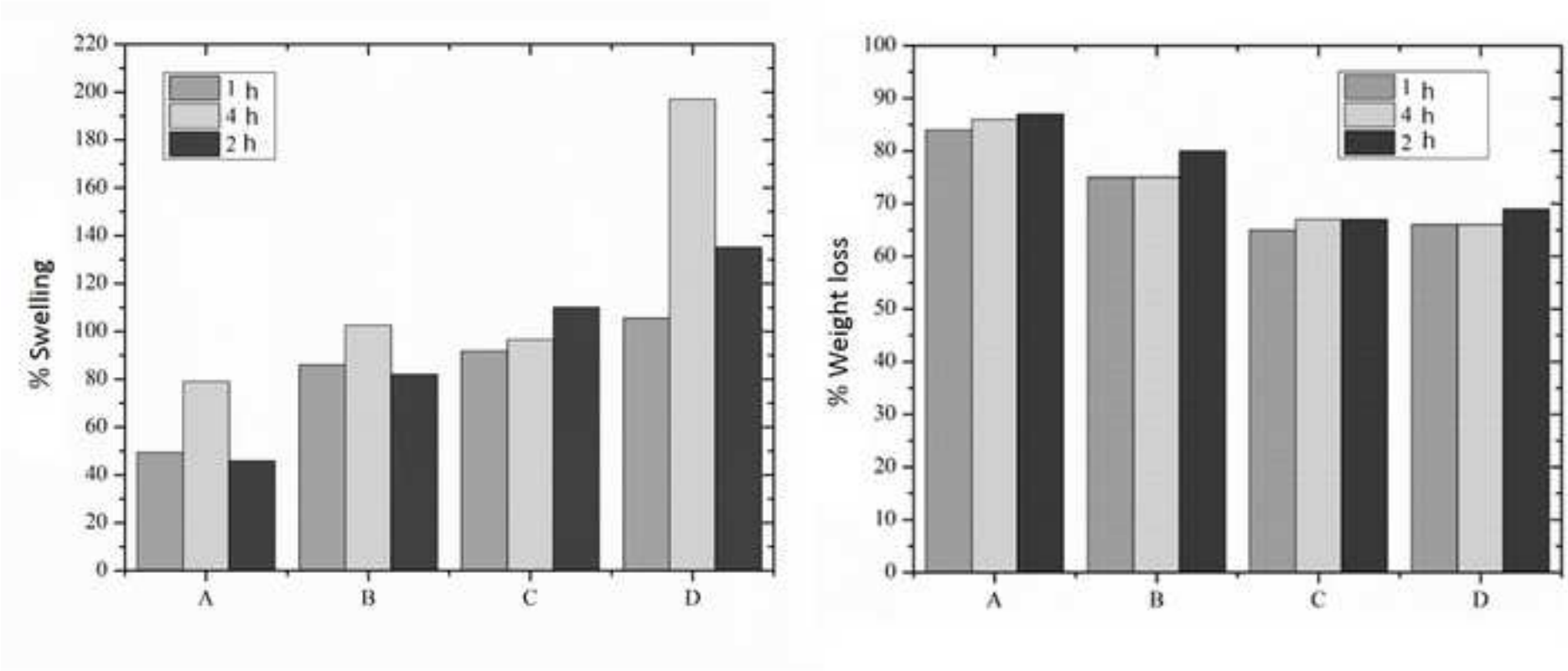
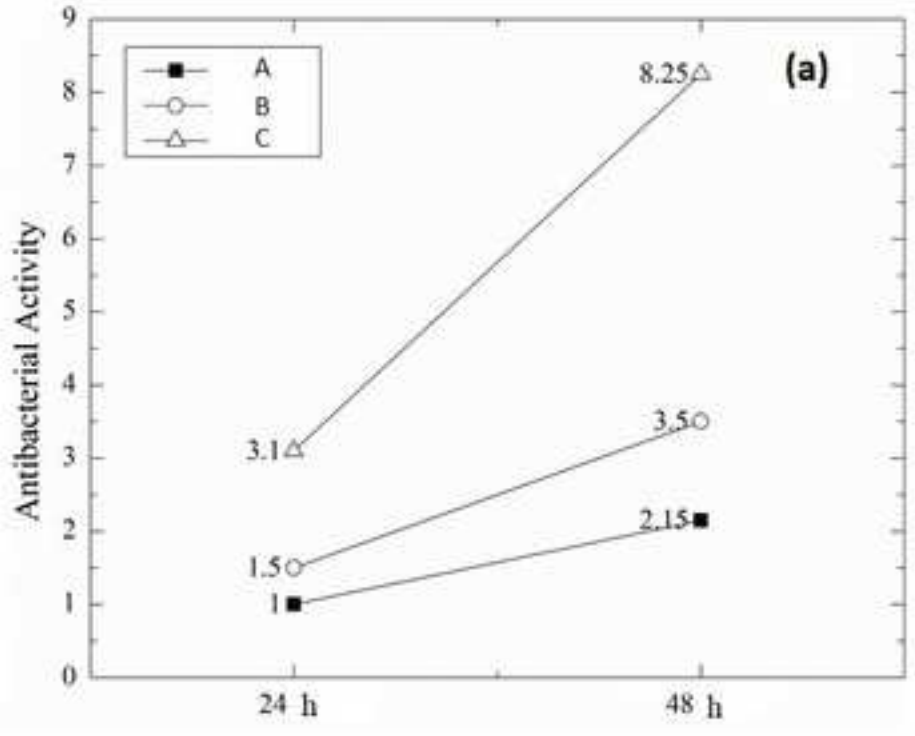

% Swelling
220
200
180
160
140
120
100
80
60
40
20
0
1 h
4 h
2 h
A
B
C
D
% Weight loss
100
90
80
70
60
50
40
30
20
10
0
1 h
4 h
2 h
A
B
C
D

Figure 6


(a)
A
B
C
Antibacterial Activity
0
1
2
3
4
5
6
7
8
9
3.1
8.25
1.5
3.5
1
2.15
24 h
48 h

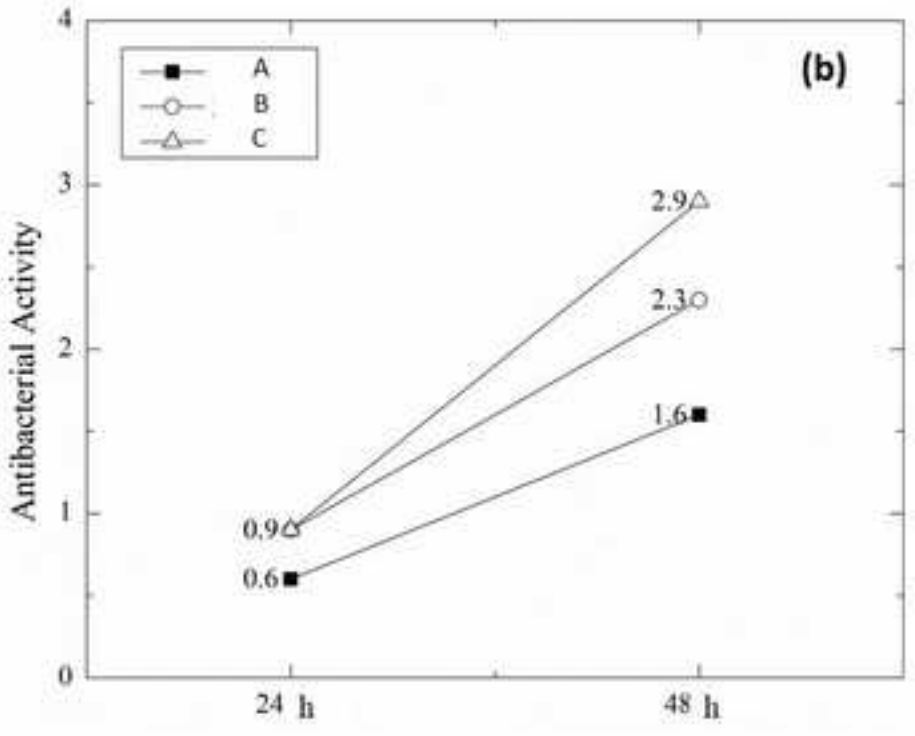
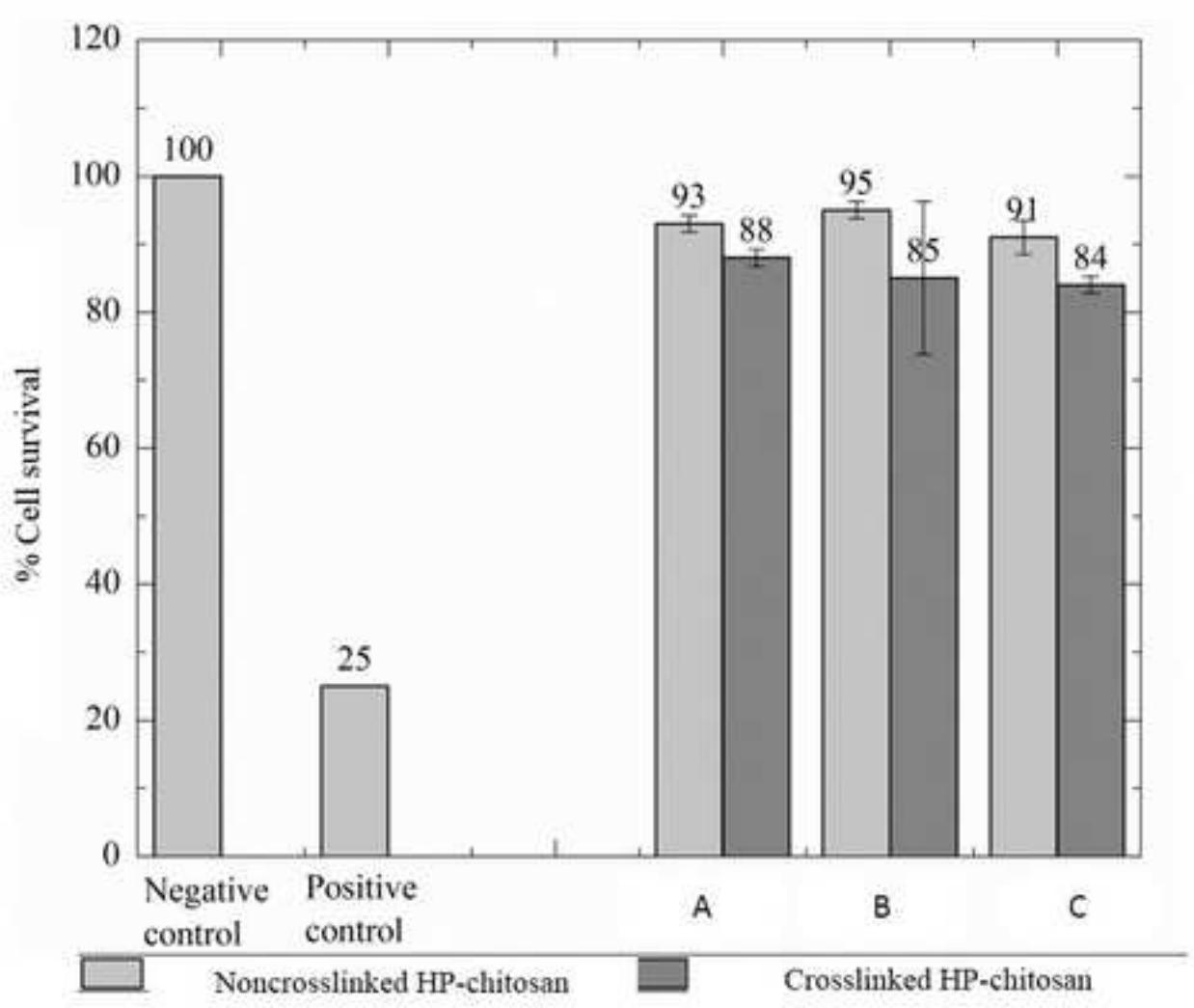

(b)
A
B
C
Antibacterial Activity
0
1
2
3
4
0.9
2.9
2.3
0.6
1.6
24 h
48 h

Figure 7


120
100
80
60
40
20
0
% Cell survival
100
25
93
88
95
85
91
84
Negative control
Positive control
A
B
C
Noncrosslinked HP-chitosan
Crosslinked HP-chitosan